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Evolution of Developmental Control Mechanisms

Piwi positive cells that line the vasculature epithelium, underlie whole body regeneration in a basal chordate

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ABSTRACT

The colonial tunicate *Botrylloides leachi* can regenerate functional adults from minute vasculature fragments, in a poorly understood phenomenon termed Whole Body Regeneration (WBR). Using *Piwi* expression (*Bl-Piwi*), blood cell labeling and electron microscopy, we show that WBR develops through activation, mobilization and expansion of 'dormant' cells which normally line the internal vasculature epithelium of blood vessels. Following a mechanical insult, these cells express *Bl-Piwi de novo*, change morphology and invade niches of the vasculature lumen, where they proliferate and differentiate, regenerating a functional organism. Mitomycin C treatments and siRNA knockdown of *Bl-Piwi* result in deficient cells incapable of expanding or differentiating and to subsequent regeneration arrest. Last, we find similar transient mobilization of *Piwi*$^+$ cells recurring every week, as part of normal colony development, and also during acute environmental stress. This recurrent activation of *Piwi*$^+$ cells in response to developmental, physiological and environmental insults may have enabled the adaptation of colonial tunicates to the imposed varied conditions in the marine, shallow water environment.

Introduction

Natural events of *in vivo* organ neogenesis by adult stem cells are rare in chordates. Proper regeneration of an organ or an organism, requires the combined 'collaborative' networking performance of: (a) stem cells that provide the building blocks (Brockes and Kumar, 2005); (b) development of a widespread 3D vascular network that meets nutrient and oxygen demands (Williams and Sebastine, 2005); (c) extracellular matrix that provides the adhesive milieu for tissue/organ shapes (Robin and Nadeau, 2001); and (d) positional information that facilitates the regenerative organ's proper outline (Brockes, 1997). Developing such a functioning network involves a complex temporal and spatial interplay of molecular cascades, cellular, chemical, and mechanical stimuli. It is therefore not surprising that whole body regeneration events are few and mostly restricted to basal metazoans like sponges (Henry and Hart, 2005), cnidarians (Bosch and David, 1987), and flat worms (Baguñà et al., 1989). An exception is the phenomenon of Whole Body Regeneration (WBR) in colonial botryllid ascidians (Brown et al., 2009; Oka and Watanabe, 1957, 1959; Rinkevich et al., 1995, 1996; Rinkevich et al., 2007a,b; Voskoboynik et al., 2007). WBR, as recognized at present, stems from circulating cells that enter the regeneration process through an unknown mechanism. A fully functional adult regenerates within 10–14 days from any arbitrarily isolated single fragment (0.1 mm) of blood vessel, containing few blood cells. Previous results revealed that this WBR event represents a novel mechanism of regeneration by several fundamental criteria, including epimorphic type of body restoration without blastema formation and the generation of multi-foci, instead of a single restoration center (Rinkevich et al., 2007b).

When we look at the yet unknown pathways, patterns, and rules, which impose limited regeneration on vertebrates, WBR in basal chordates emerges as an accessible model system for studying the basic evolutionary routes of regeneration, in an assay that follows regeneration from a single excised vasculature and few adult somatic cells. WBR is imperatively associated with the participation and activation of pluripotent stem cells. Here, we aim to trace the identity of cells underlying this regeneration process in the Mediterranean species *Botrylloides leachi*.

Materials and methods

Animal husbandry

Botryllid ascidians are shallow water encrusting colonial marine organisms. Once the planktonic tadepole-like larvae has settled and metamorphosed into a benthic founder adult (zooid), a colony develops through weekly cyclical and synchronized astogenic cycles (called blastogenesis, made up of four major stages, A–D). During blastogenesis, 1–3 new buds develop from the body wall of each parental zooid. Each cycle ends in a massive apoptotic event of all

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parental zooids concurrent with the fast development of the buds to the zooid stage (stage D, also called the 'takeover' phase). Colonies may consist of up to thousands of genetically identical modules (zooids, Fig. S1A), each 2–3 mm long and embedded within a semi-translucent gelatinous matrix, the tunic. Groups of zooids are arranged in systems connected to each other through a delicate, one cell thick vasculature network from which pear-shaped extensions (ampullae, Fig. S1B) fringe the colony.

Colonies of *B. leachi* and *Botryllus schlosseri* used here were collected from shallow waters along the Israeli Mediterranean coast and Monterey, CA, USA (Voskoboynik et al., 2007). Colonies were carefully peeled-off the underlying surfaces of stones by industrial razor blades. To minimize tissue damage, each peeled material included an attached thin layer of calcareous substrate. Isolated colonies were individually tied with fine cotton threads onto 5×7.5 cm glass slides and cultured in 17 l tanks in a standing seawater system (Rinkevich and Shapira, 1998). Within several days, the colonies glided, completely or partially, from their original calcareous substrate onto the glass slides. Colonies and their substrates were cleaned weekly with industrial razor blades and fine brushes.

Regeneration assay

Under a dissecting microscope, marginal ampullae and fragments of blood vessels were separated from colonies growing on glass slides, using an industrial razor blade and a fine tungsten needle. Then, dissected colonies were removed from the slides and attached onto other slides. The leftover blood vessel fragments were cut into smaller fragments using a fine tungsten needle and left to regenerate in Petri dishes containing filtered seawater. Fragments were monitored daily under a dissecting microscope and photographed with a Supercam camera (Applitec, Holon, Israel).

Morphologically, WBR starts with an opaque mass of blood vessels within the tunic matrix, along with a new circulatory system that is gradually being established within each regenerating fragment. Three distinctive early WBR phases were identified (Rinkevich et al., 2007b): Phase I (1–3 days post separation [dps]), formation of a new microenvironment through restructuring of the vessel architecture. The vascular epithelium detaches from the tunic and the vasculature lumen divides into small compartments ('regeneration niches'). Phase II (3–5 dps), simultaneous creation of multiple regeneration foci, started by homing of pluripotent cells into regeneration niches, aggregation and their subsequent proliferation. During phase III (5 dps and thereafter), a developmental race culminates in the maturation of a single bud to a functional filter-feeding adult ascidian-zooid.

Sequencing of Bl-Piwi

Total RNA was isolated from regenerating vascular fragments using RNeasy Mini or Midi kits (Qiagen). A cDNA fragment of 380 bp was amplified using degenerate oligonucleotide primers (forward: GCGGATCGTCGTCTACMGNGAYGGNGT; reverse: AGGCCAGCTTGTGG-GCRTANWRRCA) designed in accordance with the conserved PIWI domain. 5′ and 3′ RACE were used to obtain an open reading frame of 1650 bp. PCR products of *Bl-Piwi* were cloned into pDrive cloning vector using Qiagen PCR cloning it. Sequences related to *Bl-Piwi* were identified with BLAST. All sequences used for multiple alignments were obtained from the EMBL Nucleotide Sequence Database http://www.ebi.ac.uk/embl).

In situ hybridization

B. leachi colonies, colonial fragments containing blood vessels, and regenerating fragments were fixed overnight in 4% paraformaldehyde, gradually dehydrated in methanol (70–100%), embedded in paraffin wax and cut into 4–5 μm sections. A 570 bp clone corresponding to *Bl-Piwi* was used to obtain sense and antisense DIG-labeled RNA probes that were synthesized using the DIG RNA labeling kit (SP6/T7, Roche). *Bl-RAR* sense and antisense probes were obtained from an amplified 808 bp fragment corresponding to RAR from *B. leachi* (Rinkevich et al., 2007b). *Bl-Piwi* mRNA expression was assessed on naive blood vessels (control colonies at blastogenic stages A–C, growing under routine maintenance conditions) and regenerating ramets of *B. leachi* at sequential stages of regeneration (Rinkevich et al., 2007b), using RNA *in situ* hybridization on paraffin-embedded tissue sections. Hybridization of probes to tissue sections was performed according to Breitschopf (Breitschopf et al., 1992) for paraffin-embedded tissue. DIG-labeled RNAs on samples were revealed using anti-DIG antibody (Roche). Samples were bserved under Leica DMIRE2 inverted microscope and photographed with a Leica FX300 camera.

Polyclonal antibody production

Rabbit anti *Bl-Piwi* antibody was produced by Genemed Synthesis Inc. (http://www.genemedsyn.com). A 15 amino acid peptide corresponding to RDQSKARDFATKFNEVSRC was chosen based on its expected immunogenic properties. The peptide was synthesized and conjugated with chicken albumin and two rabbits were immunized. Following three bleeds, total antiserum was collected from the rabbits on day 70. Both sera gave similar results. Pre-immune serum was checked for reactivity revealing no expression pattern (unpublished observation; R.Y.).

Histology and immunohistochemistry

Naïve and regenerating blood vessels were fixed in Bouin's solution for 1–2 h, dehydrated in graded ethanol series (70–100%) and butanol and embedded in paraffin wax. Cross serial sections (4–5 μm) were cut by hand-operated microtome (Leica, Nussloch, Germany) and stained with alum hematoxylin and eosin for general morphology or with reagent Schiff (Feulgen reaction for DNA, Lillie, 1965).

For immunostaining, sections were attached to SuperFrost Plus microscope slides (Menzel-Glazer), dewaxed and antigen was retrieved (microwave, 480 W, 30 min in 10 mM citrate buffer [600 ml, pH 6.0]). After a 5 min cooling period, 1 l of distilled water was added and the slides were incubated for additional 10 min at room temperature, followed by several washes with TBS. Nonspecific binding sites were blocked by incubation in 1% BSA (MP Biomedicals) in 50 mM TBS for 14–16 h at room temperature. The slides were washed with TBS for 5 min, and stained with one of the following: rabbit anti *Bl-Piwi* polyclonals (1:6000 in TBS containing 1% BSA); rabbit anti *Bs-Pl10* polyclonals (1:4000 in TBS containing 1% BSA; 19); mouse anti *PCNA* monoclonal (Dako; 1:100 in TBS containing 1% BSA); rabbit anti actin polyclonal (Santa Cruz; 1:100 in TBS containing 1% BSA) for 2 h at room temperature, followed by 14–16 h incubation at 4 °C. After washing with TBS for 5 min×3, alkaline phosphatase-conjugated goat anti-rabbit (Jackson laboratories; 1:10,000 in TBS containing 1% BSA) was added as secondary antibody. Sections were incubated for 1 h–1.5 h and washed with TBS for 5 min×3. Staining reaction employed a BCIP/NBT color solution. For fluorescent staining, Cy2 or Cy3-conjugated goat anti-rabbit antibody was used (Jackson laboratories; 1:100 in TBS containing 1% BSA). For detection of nuclei, DAPI was dissolved in TBS to a concentration of 200 μg/ml and was incubated with slides for 10–15 min, followed by an additional 5 min wash. Control and experimental sections were mounted with Hydromount (National Diagnostics) or Fluoromount-G (SoutherBiotech) and photographed with Leica FX300 camera.

Western blot analysis

Western blot analysis was performed as described previously (Rosner et al., 2006).

Blood cell collections

Sub-clones of *Botrylloides* colonies at blastogenic stages A–C, each consisting of 10–40 zooids (2–3 colonial systems), were cleaned by soft brushes under a stereomicroscope (SZ-STS, Olympus Optical Co., Germany) and transferred to 35 mm sterile Petri dishes containing 3 ml tunicate culture medium (TCM) without fetal calf serum or antibiotics.

Under the stereomicroscope, small incisions were made in peripheral ampullae using the tip of a sterile needle attached to a 1 ml syringe. Incisions were made also in large blood vessels, allowing the blood cells and hemolymph to hemorrhage into the medium. Cell suspensions were collected into a 15 ml sterile tube and spun down at 1500 rpm using a swing out rotor in a table centrifuge for 10 min at room temperature. Cell pellets were re-suspended in 1 ml of TCM solution and further counted in a hemocytometer.

DiI staining of Botrylloides blood cells

CellTracker CM-DiI (Molecular Probes; C7000) is a suitable *in-vitro* labeling agent for *Botryllus* and *Botrylloides* blood cells (unpublished observations, R.Y.) as it shows no cytotoxicity, has photostable fluorescence and stains entire cells without leakage. In addition, CM-DiI remains stable after fixation and paraffin embedding procedures.

Stock solutions (50 l g/µl) were prepared by dissolving CM-DiI in ethanol, according to the manufacture's protocol. Immediately before usage, a 1–2 µl DiI stock solution was diluted in 1 ml TCM containing *Botrylloides* blood cells and incubated for 10–15 min at room temperature, then further diluted in 13 ml TCM to wash excess stain. Labeled cells were further centrifuged at 1500 rpm for 15 min. The supernatant was fully removed and the pellet was re-suspended in 20–40 µl TCM. Stained blood cells were visualized under a fluorescent microscope (Olympus BX-50, Germany) equipped with an excitation filter of 550 nm wavelength and a barrier filter of 579 nm.

Injections of Botrylloides blood cells

Recipient colonies were pre-cleaned and immersed in filtered seawater (0.2 µm). Labeled cell batches (~10 µl volume) were drawn into a glass capillary tube (KIMBLE products,KIMAX-51, U.S.A) pre-stretched using a capillary-stretcher apparatus (Narishige, Japan). Between 0.5×10^6 and 3×10^6 cells were injected intra-ampullae into a firmly attached subclone-recipient colony using a manual micro-injector and micro-manipulator (Narishige, Japan). Visualization of labeled cells inside the recipient colony was confirmed using a fluorescence microscope (Olympus BX-50).

Cell smears of Botrylloides whole blood

Drops of marine anti coagulant (MAC; 0.1M glucose, 15 mM trisodium citrate, 13 mM citric acid, 10 mM EDTA, 0.45 M NaCl, pH 7.0) were dripped onto *B. leachi* colonies under a binocular. Next, whole blood cells were extracted by puncturing marginal ampullae with a needle (27G). Blood cells were collected into a 5 ml polystyrene round bottom tube (12×75 style, BD Falcon) and centrifuged at 1500 rpm for 10 min. Cell pellets were re-suspended in 50 µl MAC and transferred into 24 tissue-culture well plates (Greiner bio-one) pre-covered with glass covers (Ø=13 mm). After 90 min of incubation, supernatants were removed and blood cells on each glass cover were fixed (10 min) with 50 µl of Bouin's fixative, diluted with artificial seawater (1:1). Then, the wells were washed with ASW followed by TBS. TBS containing 1%Triton×100 (V/V) was added and incubated for 30 min at RT. Cell smears were immunostained as described in 'Histology and immunohistochemistry' section.

RNAi treatment

Custom designed siRNAs were purchased from Ambion (www.amboin.com) based on two regions from the full-length sequence of *Bl-Piwi*. SiRNA sequence 295169: Sense: GGUGGACUAUUCUUGAAUAtt, Antisense: UAUUCAAGAAUAGUCCACCtt. siRNA; sequence 295170: Sense: CCAUCAGUUUCUUUGACUAtt, Antisense: UAGUCAAAGAAACUGAUGGtt, in addition to an unrelated control siRNA (Rosner et al., 2006). Sub-clones from different *B. leachi* genotypes were soaked for 4–7 days in 50 ml filtered seawater containing 20 nM siRNA, after which marginal ampullae were separated from colonies and allowed to regenerate for up to 16 days. Fresh medium containing 20 nM siRNA was changed every other day until termination of experiment. All RNAi treatments were conducted in a 20 °C mariculture room. Colonies and regenerating fragments were monitored daily under a dissecting microscope and photographed with a Supercam camera (Applitec, Holon, Israel).

Citral and DEAB administration

Citral (cis$^+$trans, Fluka) 60 mM stock solutions were prepared in 100% ethanol and further diluted to 20 µM and 60 µM in artificial seawater. 4-Diethylaminobenzaldehyde (DEAB, Fluka) stock solutions were prepared by dissolving 0.177gr DEAB in 1 ml DMSO in an aluminum covered Eppendorf vial. The stock solution was further diluted to 10 µM and 100 µM in artificial seawater.

Mitomycin C treatment

Mitomycin C (MMC) was purchased from Sigma. 2 mg/ml stock solutions were prepared by dissolving MMC in PBS. For preliminary experiments, colonies of *B. leachi* were immersed in filtered seawater containing 6 µM, 60 µM, 300 µM or 600 µM MMC for 24 h, followed by regeneration experiments. Both 60 µM and 300 µM MMC concentrations showed a clear phenotype and all further experiments were conducted under 60 µM MMC conditions. *B. leachi* colonies at blastogenic stage D were omitted from these experiments as ampullae of stage D colonies contain *Piwi* positive cells. Following a 24 h exposure to MMC, ampullae fragments were separated from *B. leachi* colonies and were placed in filtered seawater in a 20 °C culture room for continued regeneration. Animals and regenerating fragments were monitored daily followed by fixation in Bouin's solution.

Transmition Electron Microscopy (TEM)

Samples were fixated in EM grade 4% glutaraldehyde at room temperature, moved to 4 °C for immediate post-fixation in 1% OsO_4 in ddH2O for 1 h, washed three times in cold ddH_2O and then placed in 1% Uranyl acetate in ddH_2O for overnight. Samples were then dehydrated in graded ethanol series of 50%, 70%, 95% for 10′ each, 100% for 10′ each ×2 at room temperature, and placed in Propylene Oxide (PO) for15′. Infiltration of Epon resin was performed by submerging samples first in 1:1 PO/Epon for 1 h, followed by 1:2 PO/Epon for overnight and then 100% Epon for 2–3 h. Samples were then placed in molds labeled and filled with 100% Epon and allowed to polymerize in a 65 °C oven for 24 h. Ultrathin sections (80–90 nm) were prepared with a diamond knife (Diatome) and an ultramicrotome (Ultracut, Leica), picked up on 300 mesh nickel grids (Polysciences Inc.) and stained with uranyl acetate and lead citrate. The sections were analyzed using a Jeol 1230 transmission electron

microscope at 80 kV. Digital photographs were taken with a Gatan Multiscan 701 camera.

Immunolocalization for EM

Samples were collected and fixed in 4% paraformaldehyde with 0.05% glutaraldehyde in phosphate buffer, washed several times in PBS and infiltrated in 2.3 M sucrose overnight at 4 °C. Samples were then mounted on pins for cryo-ultramicrotomy and frozen in liquid nitrogen. Ultrathin cryosections (80–90 nm) were prepared with a diamond knife (Diatome) at 130 °C using an ultramicrotome (Ultracut; Leica) equipped with a cryosectioning chamber. Thawed cryosections were transferred to Formvar- and carboncoated EM grids (Nickel) with a drop of 2.3 M sucrose. Sections were blocked with 1% BSA in PBS for 1 h and incubated with a rabbit antibody to *Bl-Piwi* (see polyclonal 10 antibody production) for 2 h at room temperature, followed by incubation with protein A gold conjugates (15 nm gold particles) for 1 h. Sections were then counterstained with 0.5% uranyl acetate in 2% methylcellulose for 10 min on ice. Immunogold-labeled sections were visualized by a Jeol 1230TEM at 80 kV. Digital photographs were taken with a Gatan Multiscan 701 camera.

Results

Piwi$^+$ cells line closely with the vasculature epithelium

The circulatory system of *B. leachi* is a ramifying vasculature made of a single squamiform epithelial layer and hosts about 10 various blood cell types (Cima et al., 2001). Previous observations (Brown et al., 2009; Oka and Watanabe, 1957, 1959; Rinkevich et al., 1995) showed that vasculature fragments initiate WBR from aggregations of cells, appearing 2–3 days post separation (2–3 dps) within lumen compartments (termed 'regeneration niches'; Rinkevich et al., 2007b). However, it is unclear whether these cells originate from differentiated blood cells reprogrammed during WBR, whether they are a subpopulation of rare circulating stem cells, or cells that originate from the ampullar epithelium. To pinpoint their identity and location, we sequenced *Bl-Piwi*, an orthologue to the PIWI family of proteins, and a stemness marker (Brown et al., 2009; Carmell et al., 2007; Cox et al., 1998; Reddien et al., 2005). Both nucleotide and amino acid analyses of the *Bl-Piwi* sequence revealed conserved PIWI and PAZ domains, which were present in all PIWI/Argonaute family members (Fig. S2A, outlined in blue and red lines respectively). The phylogenetic affiliation of *Bl-Piwi* shows a close similarity to *Seali*, a PIWI family member from *Strongylocentrotus purpuratus* and is clustered with vertebrate *Piwi* like 2 proteins (Fig. S2B, red lines).

The expression pattern of *Bl-Piwi* mRNA was assessed on naive blood vessels (blood vessels within unharmed colonies at blastogenic stages A–C) and regenerating vasculature fragments from *B. leachi* at sequential stages of regeneration (Rinkevich et al., 2007b), using RNA *in situ* hybridization on paraffin-embedded tissue sections. *Bl-Piwi* mRNA showed no detectable staining in either blood cells or the surrounding epithelium of naive blood vessels (Fig. 1A). However, at 2 dps, we recorded *Bl-Piwi* expression in cells which were in contiguity with the epithelium and portrayed a cell size of 10–12.5 μm (Fig. 1B, arrowheads). *Bl-Piwi* expression thereafter was also present in same-size cells within blood vessel lumens (Fig. 1C). *Piwi$^+$* cell numbers increased within lumens of blood vessels as regeneration proceeded, colonizing all regeneration niches (Fig. 1D, arrowheads). A polyclonal antibody generated against *Bl-Piwi* (Fig. S3) was then employed to assess protein level expression on sections of naive and regenerating blood vessels at sequential stages of regeneration. Consistent with RNA expression patterns, *Bl-Piwi* protein was not detectable within blood cells or surrounding epithelia of naive blood vessels (Fig. 1E). However, at 2 dps *Bl-Piwi* protein was expressed within cells attached to the ampullae epithelium (Fig. 1F, arrowheads). From 2 dps to 10 dps (Figs. 1G,H,I), *Piwi$^+$* cell numbers consistently increased within the lumens of blood vessels from 124 cells/mm^2 at 2–3 dps to 457 cells/mm^2 at 6 dps and 1073 cells/mm^2 at 10 dps (Fig. 1I). Following the expression of differentiation genes (Figs. S4A–H) and the development of functional zooids, *Piwi* expression disappeared from the vasculature epithelium and *Piwi$^+$* cell numbers within the circulation of fully restored colonies plummeted to basal rates (Fig. 1I).

To better understand the morphology and location of *Piwi$^+$* cells, naive blood vessels and regenerating fragments were processed for transmission electron microscopy. In naive blood vessels, we observed distinctive flattened cells, 10–12 μm in diameter (Fig. 1J, red arrows), termed here as 'inner cells', which were not part of the epithelial layer but lined the vasculature epithelium on its luminal side (Fig. 1J, white arrows). At 2–3 dps the surrounding epithelia appeared morphologically unaffected, but the 'inner cells' were orienting away, detaching from the epithelial embedment (Fig. 1K, red arrows), subsequently homing into regeneration niches (Fig. 1L,M).

Using the generated *Bl-Piwi* polyclonal antibody, immuno-TEM of *Bl-Piwi* protein was employed on serial sections from three different regenerating fragments per each time point (0 dps, 2–3 dps and 6 dps). Consistent with light level observations, at the time point of 2–3 dps, gold particles were first observed, restricted to 'inner cells' which were attached to the epithelium (Fig. 1N, Fig. S5A) and to 'inner cells' detaching from the surrounding epithelium (Fig. 1O, Fig. S5B), but were absent from the vasculature epithelium proper. At 6 dps, gold particles were also found in 'inner cells' (Fig. 1P, Fig. S5C) and cell aggregates (Fig. 1Q, Fig. S5D) within the lumens of most regeneration niches.

We have noted that while in early stages of regeneration, *Piwi* is expressed in single cells (Figs. S6A–C). During later steps of regeneration and throughout the cellular expansion phase, *Piwi* is also expressed within cell aggregates (up to 11 cells/aggregate; Figs. S6D–F) which appear to reside in close proximity to the surrounding blood vessel epithelium (Figs. S4D–F, white arrowheads), and within lumens (Figs. S6G–H, yellow arrowheads).

Previous results estimated the frequency of circulating stem/progenitor cells within the subfamily Botryllinae to be 2×10^4–10^5 cells (Pancer et al., 1995; Simon-Blecher et al., 2004; Laird et al., 2005). We checked if such rare circulatory cells are accountable for WBR by repeatedly fragmenting blood vessels down to single blood vessel fragments (each containing 100–200 blood cells). Sixty-one fragments (each containing 1–25 blood vessels) were isolated from naive *Botrylloides* colonies and allowed to regenerate. Functional zooids developed from all fragment sizes, including single blood vessels ($n=11$, Figs. 2A–D), which regenerated functional zooids after 18 days, seven days longer than large-size fragments (Fig. 2E). Single blood vessel fragments developed early-stage buds at 14 dps (Fig. 2E) and revealed numerous *Piwi$^+$* cells (Fig. 2D, arrowheads), implying for the widespread accountability of *Piwi$^+$* 'inner cells' within all blood vessels and ampullae of the colony vasculature. In parallel, we tested the origin of *Piwi$^+$* cells by infusing host colonies with DiI-labeled cells from isogeneic donor *Botrylloides* colonies (Fig. 3A, Table S1). Analysis of recipient blood vessels showed DiI staining within numerous hemocytes (Fig. 3A) including lymphocyte-like cells previously regarded as stem cells (Cima et al., 2001). Recipient blood vessels ($n=19$) were then excised and allowed to regenerate for 6–7 days, then sacrificed for histological sections and analyzed for dual, DiI label and *Piwi* expression (Table S1). Recipient fragments developed with the idiotypic WBR morphology and cytology (Rinkevich et al., 2007b). Briefly, opaque blood vessel masses were formed within 7 dps (Fig. 3B) and within them, regeneration niches housed early-stage cell aggregates (Fig. 3C). DiI-labeled blood cells within regenerating fragments were detected in multiple sites, as single cells proximal to the vasculature epithelia (Figs. 3D–G, orange

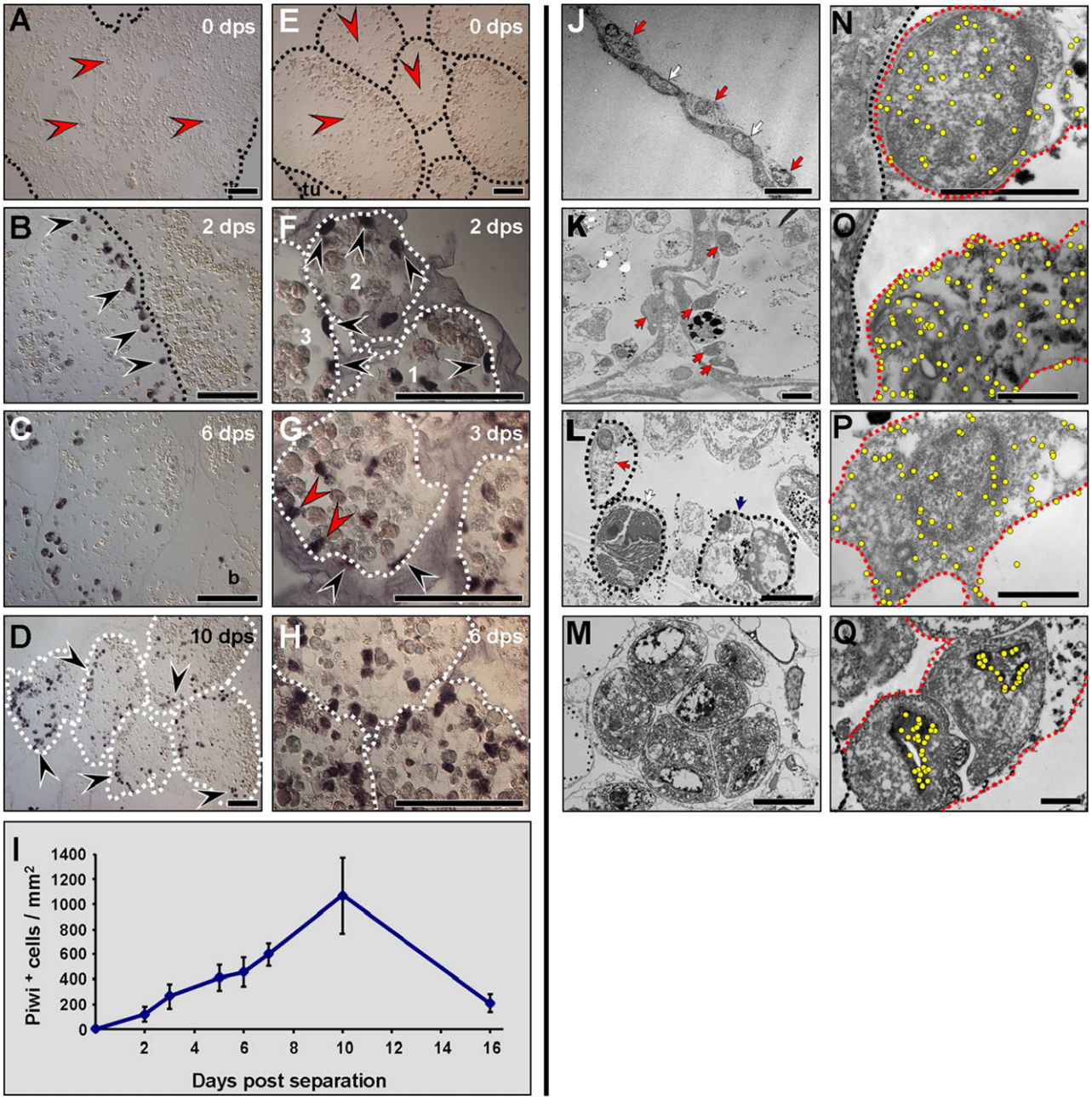

Fig. 1. Localization of *Piwi*$^{+}$ cells during whole body regeneration. *In situ* hybridization of *Bl-Piwi* mRNA (A–D), immunostaining of *Bl-Piwi* protein (E–H), Transmission Electron Microscopy (TEM) of regenerating blood vessels (J–M) and immuno-TEM of *Bl-Piwi* protein (N–Q). Naive blood vessels show absence of *Bl-Piwi* mRNA (A) and protein expression (E) within vessel's epithelia and blood cells (A,E, red arrows represent macrophage cells). At 2–3 dps, *Bl-Piwi* mRNA and protein is first recorded in 'inner cells' lining the epithelium of blood vessels on their luminal side (B,F arrowheads, G, black arrowheads). 'Inner cells' juxtaposed to the surrounding epithelia, invade the ampullae lumens (G, red arrowheads; three regeneration niches [F1–3]) and acquire a round morphology (C and H). Systemic distribution of *Piwi*$^{+}$ cells at 6 to 10 dps (D, arrowheads). (I) *Piwi*$^{+}$ cell numbers were counted from consecutive sections of five different regenerating fragments at each indicated time point. (J–M) TEM study reveals location of 'inner cells'. A reposed blood vessel displaying three 'inner cells' (J, red arrows), lining the blood vessel epithelium (J, white arrows). (K) At 2 dps, 'inner cells' orient and detach from the vessel epithelium (K, red arrows) and subsequently are detected within vessel's lumen (L, red arrow; white and blue arrows indicate macrophage-like cells). (M) A cell aggregate consisting of six cells after 6 dps. (N–Q) Immuno-TEM of *Bl-Piwi*; Gold particles (highlighted as yellow dots) are restricted to 'inner cells'. (N) Regenerating fragment at 2 dps; gold particles are localized to 'inner cells' (outlined by red dotted-line) adjacent to the blood vessel's wall (outlined by black dotted-line) and are absent from underlying epithelia. (O) Regenerating fragment at 2–3 dps; gold particles are located within a detaching 'inner cell' (outlined by red dotted-line) and are absent from surrounding epithelia (outlined by black dotted-line). (P) Gold particles within a circulating 'inner cell' (outlined by red dotted-line) at 6 dps. (Q) Gold particles stain the nuclei of a cell aggregate (outlined by red dotted-line) at 6 dps. b, bud; dps, days post separation; tu, tunic. Scale bars: A–H = 100 μm; J–M = 5 μm; N–Q = 1 μm.

arrowheads) and in cellular assemblages at blood vessels' centers (Figs. 3H–K, orange arrowheads). *Piwi*$^{+}$ cells were also present in proximity to the vasculature epithelia as well as within the vessel's lumen (Figs. 3D–K, green arrowheads). We counted the numbers of blood cells within recipient fragments co-stained with DiI and *Piwi* protein. Out of 2425 DiI-labeled cells counted, only nine (0.003%) showed a dual DiI/*Piwi* staining (Table S1). We then inversely counted 1700 *Piwi* positive cells within recipient fragments, of which only eight cells co-labeled with DiI (0.005%).

The use of small blades and brushes during routine cleaning of *Botrylloides* colonies ('animal husbandry' in M&M) occasionally inflicts damages on peripheral ampullae. Rehabilitation of such damaged ampullae during the husbandry of donor colonies, therefore, may be accountable for the existence of small numbers of circulating

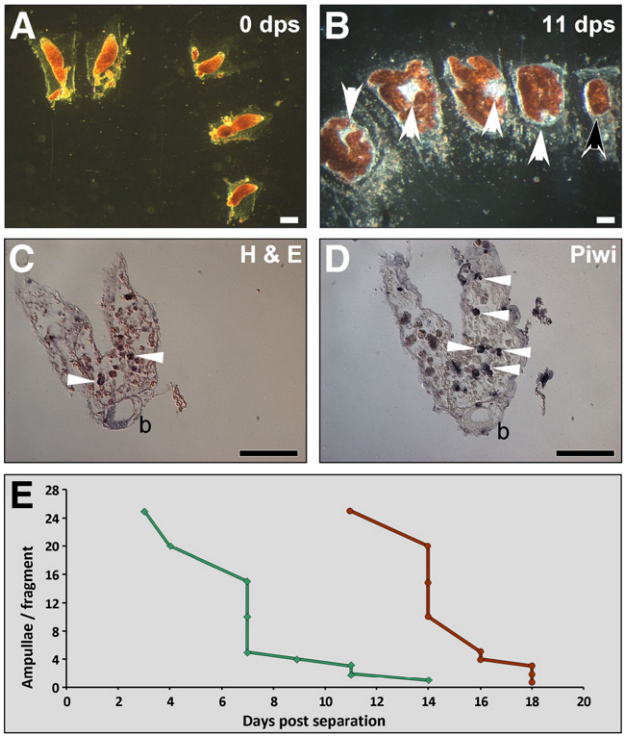

Fig. 2. Whole body regeneration from single blood vessel fragments involves *Piwi*$^+$ cells. (A) Single-ampullae fragments containing 100–200 circulating cells, each, immediately after separation from *Botrylloides leachi* colonies. (B) single — (black arrow) and multiple — (>2, white arrows) blood vessel fragments at 11 dps. (C) Hematoxylin and eosin section of a single blood vessel fragment at 14 dps showing a developing bud and multiple cell aggregates within the lumen (arrowheads). (D) Same fragment as in C. Immunostaining of a single blood vessel fragment showing *Piwi*$^+$ cells within the blood vessel lumen (arrowheads). (E) Tempo of regeneration (green — first bud appearance; brown — development of a functional zooid) is associated with fragment size. b, bud; dps, days post separation; H&E, hematoxylin and eosin. *Piwi* positive cells represent protein level expression. Scale bars = 100 μm.

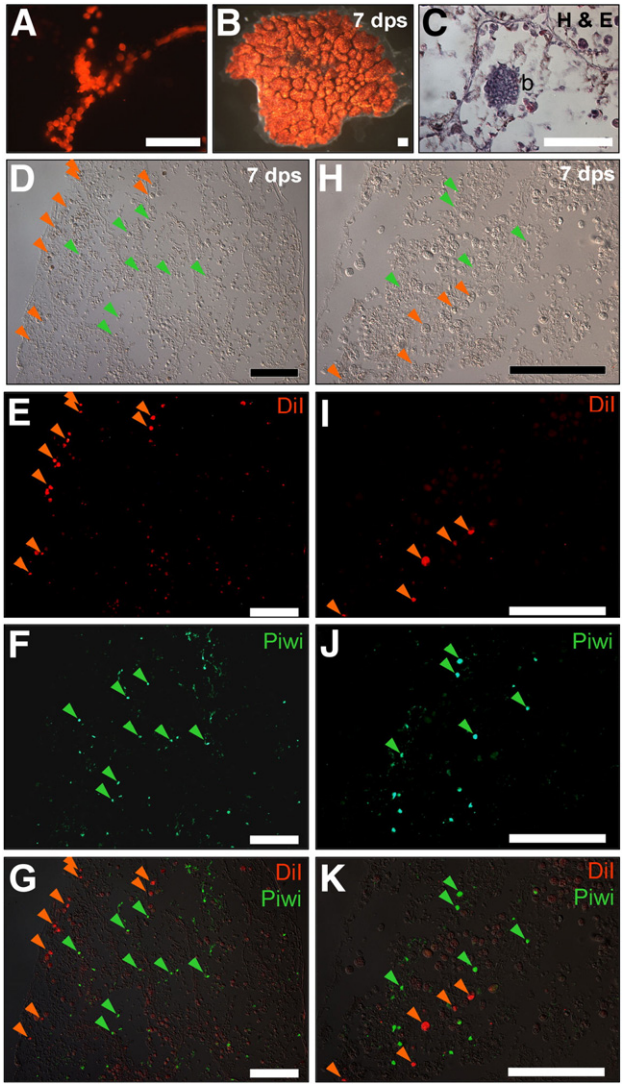

Fig. 3. *Piwi*$^+$ cells are not of circulatory origin. (A) A recipient colony infused with DiI-labeled blood cells. (B,C). A recipient fragment at seven dps showing an opaque blood vessel mass (B) and a regeneration niche occupied by a cell aggregate (C). DiI-labeled cells are detected in peripheral (D–G, orange arrowheads) and central locations (H–K, orange arrowheads). D and H = bright fields. Labeled blood cells are *Piwi* negative (G,K, orange arrowheads). *Piwi*$^+$ cells are not labeled with DiI (G,K, green arrowheads). b, bud; dps, days post separation; H&E, hematoxylin and eosin. Scale bars = 100 μm. *Piwi* positivity represents protein expression.

Piwi positive cells in recipient colonies. Therefore, blood cells from same donor colonies were examined for *Piwi*$^+$ cell percentages. Results revealed a similar dual label percentage (0.005%, 4125 cells counted; Mann–Whitney test $p \gg 0.05$; Table S1). Above results, therefore, refute the suggestion for a circulating stem or progenitor cell (in undisturbed *Botrylloides* colonies) as the primary source for WBR and collectively contend that *Piwi*$^+$ 'inner cells' are the initial cell source for WBR.

We next examined the cell cycle status of *Piwi*$^+$ cells during regeneration by employing PCNA antibody. During early WBR stages (2 dps), PCNA predominantly stains *Piwi*$^+$ cells attached to the epithelium (Figs. 4A–D, arrowheads) whereas later on (6 dps), PCNA also stains *Piwi*$^+$ cells within the lumen (Figs. 4E–H, arrowheads). At 6 dps, 75.5% of all circulating *Piwi*$^+$ cells were stained for PCNA ($n = 480$), with a progressive decline thereafter of *Piwi*$^+$ cell proliferation (52.5% [$n = 450$] at 8 dps, 21% [$n = 420$] at 10 dps and 5% [$n = 190$] at 14 dps respectively). Therefore, we chemically impeded cells from entering the cell cycle by using Mitomycin C (MMC) and asked whether it would lead to a decline in *Piwi*$^+$ cell numbers. *B. leachi* colonies were temporarily exposed to MMC and then their ampullae were separated and allowed to regenerate. MMC treated fragments displayed blood vessel shrinkage, movements and anastomoses including a sluggish blood flow, all of which are morphological characteristics of early regeneration steps (Rinkevich et al., 2007b). However, regeneration within MMC treated fragments did not advance (compare control fragments [Figs. 4I–L] to MMC treated fragments [Figs. 4M–P]). Blood vessels in MMC treated fragments failed to coalesce or to form opaque blood vessel masses, and buds did not emerge (compare Fig. 4I, arrow to Fig. 4M), even after 15 dps (usually visible after 7 dps). Histological analysis revealed regeneration niches without buds or cell aggregates (the earliest morphological indication of WBR, compare Figs. 4J–N). We checked for cell proliferation within MMC treated fragments and found no staining of PCNA (compare Figs. 4K–4O). We then checked for *Piwi* expressions within MMC treated fragments and found *Piwi* staining restricted to 'inner cells' still attached to the surrounding epithelium. No detection of any *Piwi*$^+$ cell expansion was documented within the lumen (compare Figs. 4L–P, arrowheads), supporting the claim that circulating *Piwi*$^+$ cells arise from the *Piwi*$^+$ 'inner cell' population.

siRNA mediated knockdown of Bl-Piwi results in deficient cells and to regeneration arrest

The impact of *Bl-Piwi* knockdown on WBR was assessed using RNAi on regenerating fragments. Ampullae and blood vessels, surgically separated from *B. leachi* colonies, were soaked for up to 16 days in filtered seawater containing custom designed siRNA against *Bl-Piwi*. RNAi treated fragments at 10 dps expressed residual

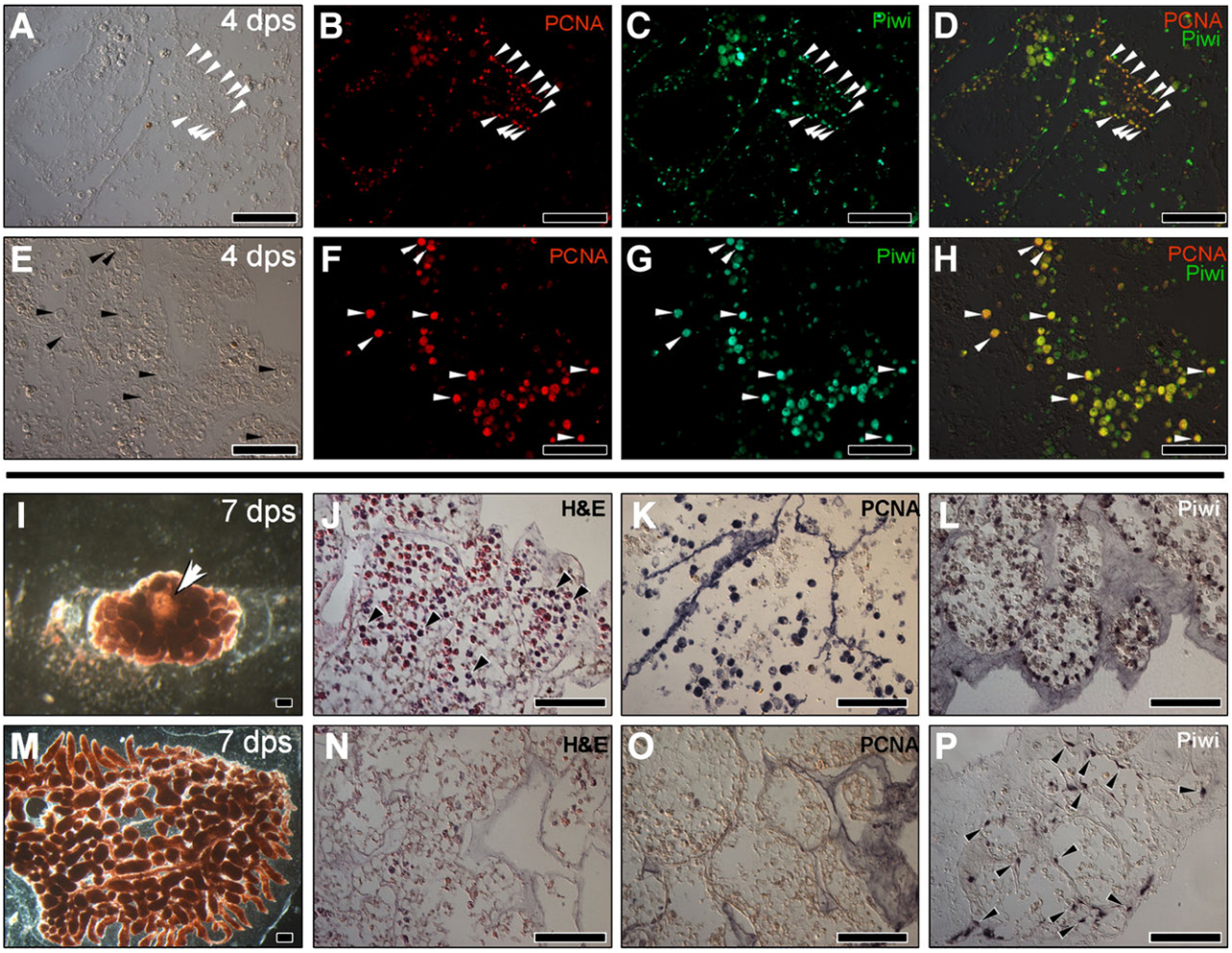

Fig. 4. Mitomycin C (MMC) eliminates circulating *Piwi*$^{+}$ cells and prevents WBR. Cells co-stained for PCNA and *Bl-Piwi* protein (arrowheads) adjacent to the epithelium (A–D) and lumen (E–H). A and E = bright fields. A control (I–L) and MMC treated fragments (M–P) at 7 dps. Lack of regenerating buds (M) and cell aggregates (N) within MMC treated fragments as compared to controls (I, arrow; J, arrowheads). PCNA (K,O) and *Bl-Piwi* (H,L) immunostaining within control and MMC treated fragments. Absence of circulating *Piwi*$^{+}$ cells in MMC treated fragments (P, arrowheads). dps, days post separation; PCNA, proliferating cell nuclear antigen; H&E, hematoxylin and eosin. *Piwi* positivity represents protein expression. Scale bars = 100 μm.

Bl-Piwi mRNA and protein levels (Figs. 5A,B) as compared to controls and in seven of the 18 cases, no staining was recorded in the vasculature epithelia, nor in lumen cells. In other cases, *Piwi*$^{+}$ cell numbers plummeted by an order of magnitude to 77 cells/mm^2 at 10 dps, compared to 1073 cells/mm^2 of same stage regenerating controls (Figs. 5A). Prolonged RNAi treatments (16 days) halted WBR completely, i.e. functional zooids were not formed (compare control fragments [Figs. 5C–F] to regenerating fragments [Figs. 5G–J]). Histological analyses revealed multiple niches within vascular lumens, however, without developing buds (compare Figs. 5D–H; buds usually appear from days 3–4). Hematoxylin–eosin (compare Figs. 5D–H) and DAPI staining (compare Figs. 5K–M to Figs. 5 N–P) showed single cells within regeneration niches with a complete absence of rounded, cell aggregates. To better understand the impact of *Bl-Piwi* RNAi we checked for any change in cellular proliferation or cellular differentiation during WBR and found a complete absence of PCNA expression within RNAi treated fragments (Fig. S7A). Differentiation genes which were normally up-regulated from early WBR stages, such as *Bl-RAR* (retinoic acid receptor) were found similarly to be absent (Fig. S7B), while Pl10 expression was restricted to other blood cell types, such as macrophage-like cells (Fig. S7C). Previously, we have found an early requirement for retinoic acid signaling in the early steps of bud formation and differentiation during WBR (Rinkevich et al., 2007b). We then chemically impeded early retinoic acid synthesis, using either DEAB (4-Diethylaminobenzaldehyde) or Citral (3,7-dimethyl-2,6-octadienal). RA-inhibition resulted in regeneration arrest (compare control fragments [Figs. 6A–C] to RA-inhibited fragments [Figs. 6D–F]). RA-inhibited fragments developed buds which appeared disorganized and undifferentiated (Fig. 6F, arrowhead). While in normal regenerating fragments, *Piwi* was not expressed within developing buds (Fig. 2D) but confined to single 'inner cells' and cell aggregates (Figs. 6G–J), in RA-inhibited fragments, tissue structures retained high levels of *Piwi* expression (Figs. 6K–N). *Piwi* expression/activity thus appears epistatic to the early expression/activity of the RA signaling pathway previously reported (Rinkevich et al., 2007b) and represents an early ephemeral cellular state which precedes cellular differentiation. Loss of *Bl-Piwi* perturbs the cellular expansion and leads to regeneration arrest.

Recruitments of Piwi^{+} *cells is a common premise to life history events of botryllid ascidians*

In the conspecies *B. schlosseri*, WBR takes place only during the 24–36 h period of blastogenic stage D in the weeklong developmental cycle of the colony astogeny (Voskoboynik et al., 2007). To evaluate any correlation between *Piwi* expression and regeneration capacity within the subfamily Botryllinae, we followed regeneration scenarios imposed on colonies of the closely related genus *B. schlosseri* at different blastogenic

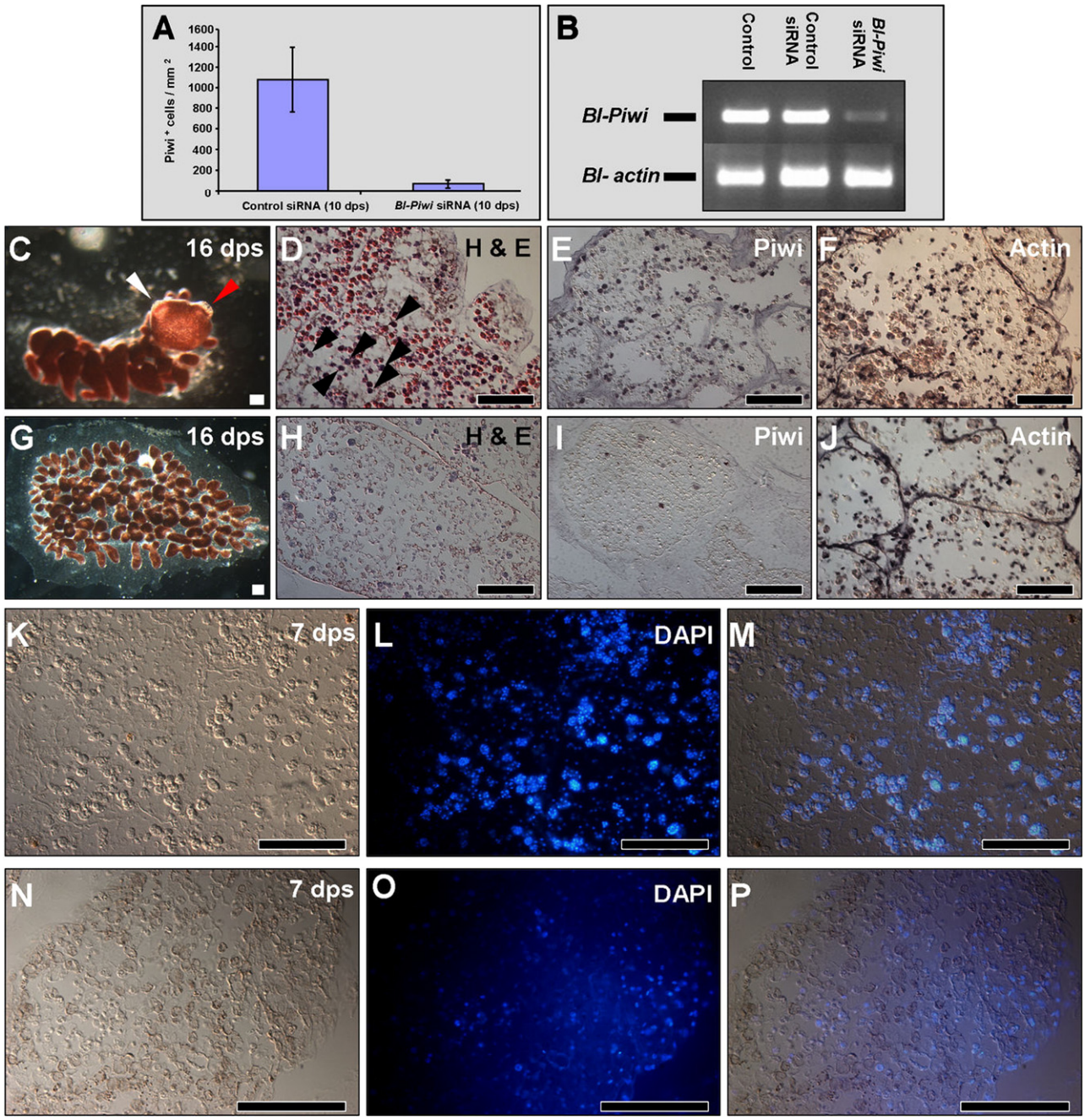

Fig. 5. *Bl-Piwi* knockdown results in early regeneration arrest. (A) Diagram depicting *Piwi*$^+$ cell numbers within control and RNAi treated fragments at 10 dps. (B) *Bl-Piwi* but not Actin (positive control) transcript level decrease in RNAi treatments. Control (C–F) and RNAi (G–J) fragments at 16 dps. A functional adult zooid (C) with branchial and atrial siphons (red and white arrowheads, respectively) as compared to undeveloped RNAi (G) treated fragment. Hematoxylin and eosin sections of control and RNAi treated fragments show numerous cell aggregates in controls (D, arrowheads) with complete absence in RNAi treatments (H). Multiple *Piwi*$^+$ cells in controls (E) and reduced numbers in RNAi treatments (I). Actin (positive control), shows similar expression within control (F) and RNAi (J) treatments. Cell aggregates within control (K–M) and absence in RNAi treatments (N–P). K, N = bright fields; L, O = DAPI; M, P = merged (bright field + DAPI). dps, days post separation; H&E, hematoxylin and eosin; DAPI, 4′,6-Diamidino-2-phenylindole dihydrochloride. *Piwi* positivity represents protein expression. Scale bars = 100 μm.

stages. Consistent with previous observations (Voskoboynik et al., 2007), fragments isolated from *Botryllus* colonies at blastogenic stages A/B which did not regenerate ($n = 1/8$, Fig. 7A) also exhibited minimal numbers of *Piwi*$^+$ cells (31–44 cells/mm^2 at 10 dps, Figs. 7B,C,M Table S2). Fragments isolated at blastogenic stage D which did regenerate (buds appeared at 4–6 dps and functional zooids within 10 days [$n = 7/9$, Figs. 7D,E]) showed a fold higher number of *Piwi*$^+$ cells within regenerating fragments (293–422 cells/mm^2 at 10 dps; Fig. 7M, Table S2), even within single blood vessel fragments (Fig. 7F, arrowheads).

Above results, led us to test whether the expression of *Piwi* represents a weekly cyclical phenomenon during blastogenesis, a process believed to be regulated by stem cells (Lauzon et al., 2002). Each blastogenic cycle possesses three major developmental stages (stages A–C) followed by a degenerative stage D (also called 'takeover'), in which a whole-organism apoptotic event coupled with the fast maturation of buds culminates in the reconstitution of the colony (Fig. 7G; Manni and Burighel, 2006). Colonies of *B. leachi* at blastogenic stages A–C, revealed no *Bl-Piwi* mRNA (Fig. 1A) or protein expression (Fig. 1E). In contrast, at blastogenic stage

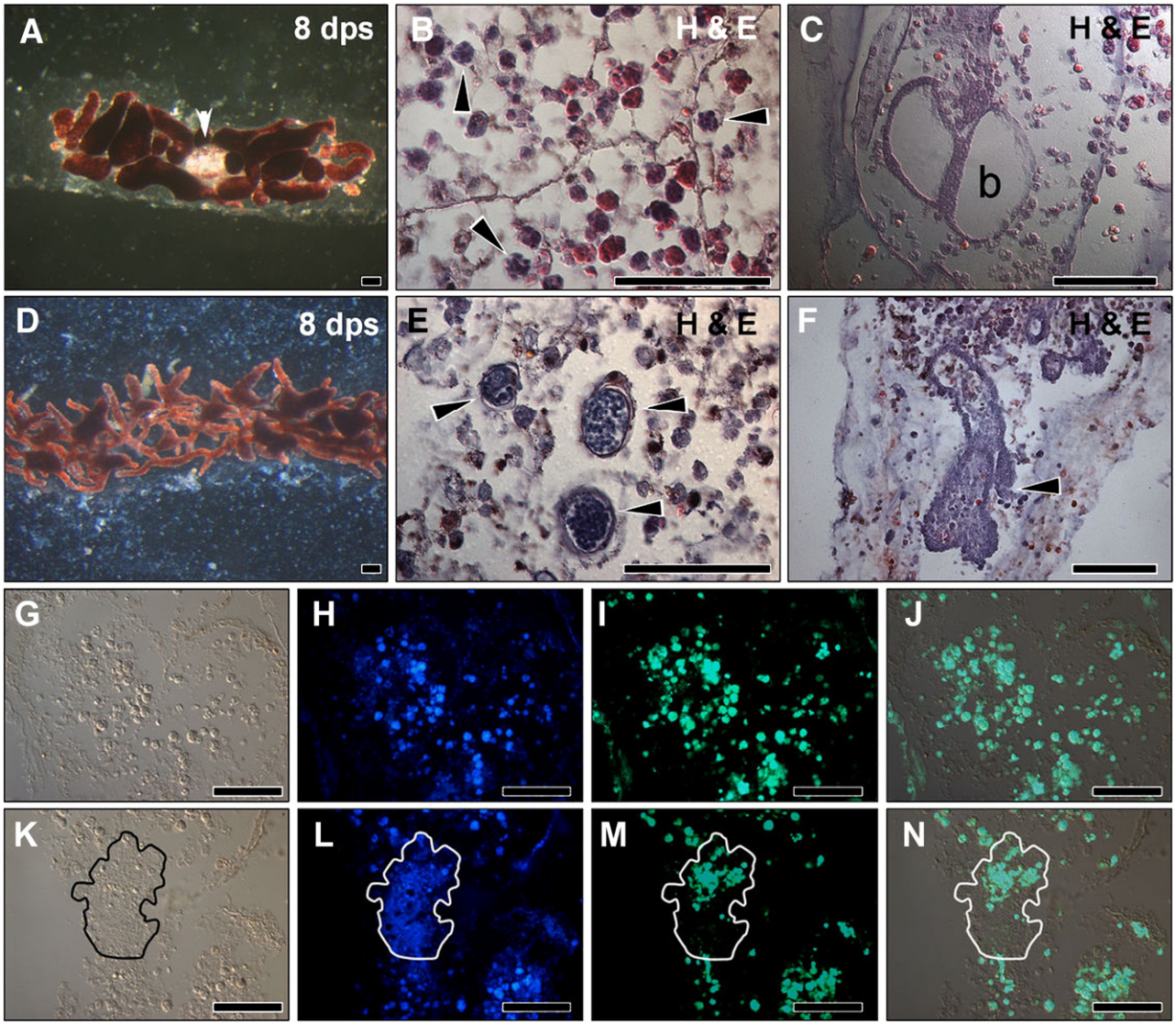

Fig. 6. *Piwi*$^+$ cells do not differentiate in RA-inhibited fragments. Control fragment, showing a highly developed bud (A, arrow) as compared to the Citral treated fragment (D) where no bud has developed. Hematoxylin and eosin control sections showing cell aggregates (B, arrowheads) and a vascular bud at organogenesis (C) as compared to a Citral treated fragment showing giant cell aggregates (E, arrowheads) and undifferentiated chaotic tissue masses within vasculature niches (F, arrowhead). (G–J): Accumulation of *Piwi*$^+$ cells within a Citral treated fragment. (K to N) An undifferentiated chaotic tissue mass (outlined) within a Citral treated fragment expressing *Piwi*. G, K = bright field; H, L = DAPI; I, M = *Piwi*; J, N = merged. B, bud; DAPI, 4′,6-Diamidino-2-phenylindole dihydrochloride; dps, days post separation; H&E, hematoxylin and eosin. *Piwi* positive cells represent protein level expression. Scale bars = 100 μm.

D, we found *Piwi*$^+$ cells in multiple blood vessel sites (191 cells/mm^2, Figs. 7H,I,M), attached to the epithelium and within the lumen of both marginal and centrally located blood vessels.

Next, we asked whether *Piwi*$^+$ cells are present in stressed or naturally regressed colonies. Colony regression (Berrill, 1951; Nakauchi, 1982), termed also 'aestivation' or 'hibernation' (Rinkevich et al., 1996), describes a physiological and morphological response of botryllid ascidians to adverse environmental changes, such as rapid rise or drop in seawater temperatures. This process involves rapid morphological resorption of all zooids and buds of the colony, leaving behind a dense 'carpet' of opaque ampullae and blood lacunae (Fig. 7J), which upon improved environmental conditions, regenerate new colonies. The morphology and histology of regressed *B. leachi* colonies collected from the wild and from our mariculture facility are both reminiscent of early WBR states. Notably, cell aggregates were present, colonizing niches within blood vessels (Fig. 7K) and accordingly, *Piwi*$^+$ cells were indeed present within numerous blood vessel sites of the colony vasculature (311 cells/mm^2, Figs. 7L,M).

Discussion

Stem cells of tunicates, including colonial botryllid ascidians, play a center role in a myriad of biological events, including aestivation, hibernation, blastogenesis, 'takeover', colonial chimerism, cellular parasitism and diverse regeneration scenarios (Berrill, 1951; Brown et al., 2009; Laird et al., 2005; Oka and Watanabe, 1957, 1959; Rinkevich et al., 1995, 2007b; Stoner et al., 1999; Voskoboynik et al., 2007). Considering their key taxonomic position at the base of the vertebrate branch (Delsuc et al., 2006), colonial tunicates are a valuable group of organisms for studying the biology of adult stem cells, a scientific discipline currently lacking identifiable stem cell markers.

The present results establish that Whole Body Regeneration (WBR) in botryllid ascidians involves activation of *Piwi*$^+$ 'inner cells' which commence the road to a full regeneration through their transient activation of *Piwi*, their mobilization and expansion within blood vessels. Moreover, animals regularly activate *Piwi*$^+$ 'inner cells' following trauma or environmental insults. A similar WBR

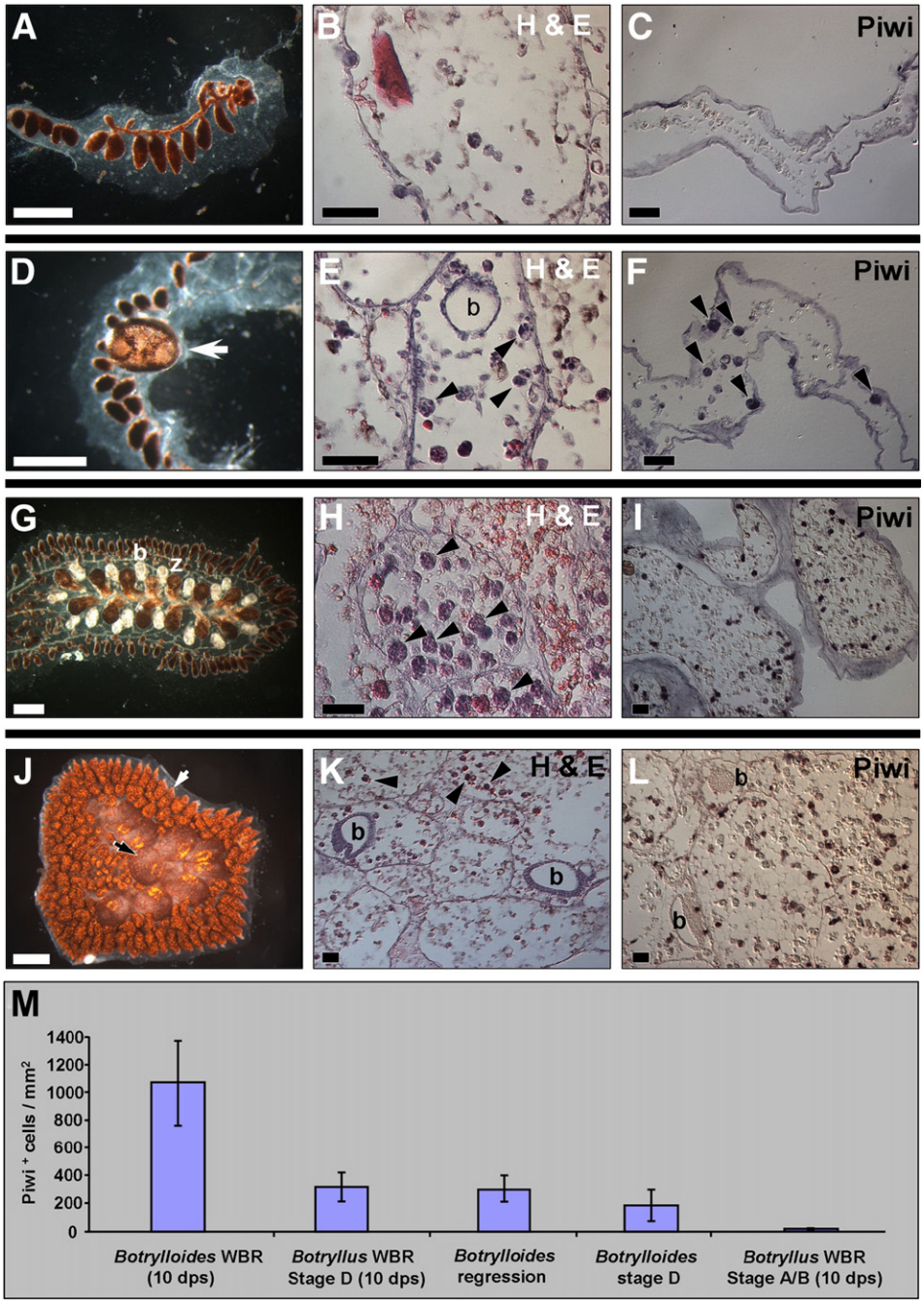

Fig. 7. *Piwi*$^+$ cell activation is ubiquitous to restoration scenarios of botryllid ascidians. (A–C) *Botryllus schlosseri* fragments taken from blastogenic stages A/B at 10 dps do not regenerate: (A) general morphology, (B) hematoxylin and eosin staining showing regeneration niches without buds, and (C) *Piwi* is not expressed above background levels within regenerating ampullae. (D–F): *Botryllus schlosseri* fragments taken from blastogenic stage D colonies at 10 dps successfully regenerate functional adult zooids (D, arrow). Hematoxylin and eosin staining documents a regeneration niche occupied by a developing bud (E) and cell aggregates (E, arrowheads). *Piwi* is expressed within regenerating ampullae from stage D colonies (F, arrowheads). (G–I): *Botrylloides leachi* colony at blastogenic stage D in which functional zooids are resorbed concomitant with maturation of buds (G). A stage D colony displays cell aggregates (H, arrowheads) and shows multiple *Piwi*$^+$ cells (I). (J–L): a regressed *Botrylloides leachi* colony exhibiting resorbed zooids (J, black arrowhead) and the formation of a 'carpet' of ampullae (J, white arrowhead). A cross section through regeneration niches that are occupied by developing buds (K,L) and reveal multiple *Piwi*$^+$ cells (L). (M) *Piwi*$^+$ cell numbers typically distinguish diverse restoration scenarios. b, bud; z, zooid; dps, days post separation; H&E, hematoxylin and eosin; WBR, whole body regeneration. *Piwi* positive cells represent protein level expression. Scale bars: A–D = 1 mm; E–L = 50 μm.

phenomenon and expression of *Piwi*$^+$ cells was recently documented in a closely related species *Botrylloides violaceus* (Brown et al., 2009) using a commercially available antibody, and may reflect a conserved attribute to colonial tunicates.

In the absence of appropriate tools to enable direct cell sorting of the *Piwi*$^+$ cells and their descendents, several independent lines of evidence collectively suggest that the potential for pluripotency resides primarily in *Piwi*$^+$ cells within the colony vasculature. These include: (1) localizations of *Bl-Piwi* mRNA and protein during WBR and in single-ampullae fragments; (2) mitomycin C treatment and knockdown of *Bl-Piwi* inhibits the expansion of *Piwi*$^+$ cells and prevents regeneration; and (3) DiI-labeled blood cells infused into regenerating isogeneic recipients are *Piwi* negative, while *Piwi* positive cells are not stained for DiI.

Piwi belongs to the evolutionarily conserved PIWI/Argonaute superfamily of RNA interference effector proteins which are essential for both self-renewal and maintenance of germ-line and somatic stem cells in diverse multicellular organisms (Brown et al., 2009; Carmell et al., 2007; Cox et al., 1998), including lower eukaryotes and plants. The

knockdown phenotype of *Bl-Piwi* during WBR demonstrates a critical and early role for *Piwi* in the expansion/self-renewal of the progenitor pool, loss of which inhibits regeneration. *Piwi* expression at onset of WBR, as in other biological events, brings to mind the participation of *Piwi* related genes in other metazoans, where it also shows a transient expression. In cnidarians, the *Piwi* homologue *Cniwi* is expressed during trans-differentiation of striated muscle cells into smooth muscle and nerve cell lineages (Seipel et al., 2004). In plants, the *Piwi* homologue *ZWILLE* is expressed during shoot meristem formation (Moussian et al., 1998), while in humans, the *Piwi* homologue *hiwi* is associated with cancer development (Qiao et al., 2002).

WBR in botryllid ascidians is more than just regeneration of body parts after a traumatic amputation. It uncovers a biologically significant pathway (Fig. S8) for activation and expansion of $Piwi^+$ cells that is re-utilized in response to developmental demands as well as to altered environmental conditions imposed on the organism. In this regard, the unexpected transient expression of *Bl-Piwi* within blood vessels at blastogenic phase D illustrates that in botryllid ascidians, the cyclical destruction of a whole generation of zooids coincides with a recurring process in which $Piwi^+$ cells are activated *de novo*, every week, as part of the normal colony astogeny. Furthermore, regeneration scenarios following *in situ* colony regressions in the wild and under laboratory settings may represent ecological manifestations of the WBR phenomenon, i.e., derived *de novo* from *Piwi* expressing 'inner cells'.

Formation of transient multicell aggregates during WBR may also be related to a wider vertebrate phenomenon, the emergence of multicell aggregates or multi-nucleated cells, in many cases formed through the process of heterotypic cell fusion during chronic inflammation, radiation, and other insults (Alvarez-Dolado, 2007; Johansson et al., 2008). The possible relationships between 'inner cells' and the cell aggregates were not addressed here. However, the observed outcomes of their temporal appearance, *Piwi* expression and their disappearance from regenerating fragments following MMC treatment or *Bl-Piwi* knockdown, all suggest that they may be formed by proliferation of $Piwi^+$ cells without proper cytokinesis.

The co-option of a stem cell activation circuitry during colony development, hibernation and diverse regeneration scenarios, may have enabled the quick adaptation to colonial life-form on shallow water hard substrates by responding to newly imposed and fast changed physiological and developmental needs. Addressing topics of WBR in model organisms is going to yield fundamental insights into metazoan biology, attesting to the ability of a single line of somatic cells to give rise *in vivo* to descendents of all germ layers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ydbio.2010.05.500.

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